Chronobiology International, 23(1&2): 393–401, (2006)

ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500521855

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THE PERIVASCULAR PHAGOCYTE OF THE MOUSE PINEAL GLAND: AN ANTIGEN-PRESENTING CELL

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The perivascular space of the rat pineal gland is known to contain phagocytic cells that are immunoreactive for leukocyte antigens, and thus they appear to belong to the macrophage/microglial cell line. These cells also contain MHC class II proteins. We investigated this cell type in the pineal gland of mice. Actively phagocytosing cells with a prominent lysosomal system were found in the pericapillary spaces of the mouse pineal gland following intravenous injection of horseradish peroxidase. The cells also exhibited strong acid phosphatase activity. Perivascular cells were immunopositive for MHC class II protein and for CD68, a marker of monocytes/phagocytes. This study verifies that perivascular phagocytes with antigen-presenting properties are present in the mouse pineal gland.

Keywords Pineal, Phagocyte, Acid phosphatase, Houseradish peroxidas, Mouse

INTRODUCTION

The mammalian pineal gland is located in the dorsal region of the diencephalon and is covered by a pial capsule. The gland consists of parenchyma, and in several species arranged in lobules, separated by interlobular spaces containing blood vessels, nerve fibers, and a few interstitial-like cells. In the parenchyma, two cell types are present: 1) hormone-secreting pinealocytes and 2) interstitial cells (Møller, 1974). Both cell types are easily distinguished in electron micrographs, since the cytoplasm of the interstitial cell has a higher electron density than the pinealocyte. In addition, interstitial cells are characterized by glial filaments; some are also immunoreactive for the glial fibrillary acid protein (GFA) (Møller

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et al., 1978a). The interstitial cells are located between the pinealocytes and are also present on the surface of the lobules bordering the perivascular spaces. Cells with an interstitial cell-like morphology are also located within the perivascular spaces. During the last decades, these cells have been classified as a special subgroup of interstitial cells designated as "perivascular phagocytes" and have been found to be antigen-presenting cells (Pedersen et al., 1993; Sato et al., 1996; Jiang-Shieh et al., 2003).

Previous investigations of pineal perivascular phagocytes were performed in the rat. Here, we examine the mouse pineal gland as the first step toward the use of knockout mice to investigate the physiological role of these antigen-presenting cells.

MATERIALS AND METHODS

Animals

Albino BALB/c/C57/B6 Tac mice (22 to 28 g) were studied. The animals were kept on a 12:12 h light-dark (LD) schedule (L on at 06:00 h), with food and water available *ad libitum*. All experiments and perfusions of animals were performed during the daytime. The experiments were conducted in accord with the guidelines of *EU Directive 86/609/EEC* and the standards of the Journal (Touitou et al., 2004) and were approved by the Danish Council for Animal Experiments.

Electron Microscopy

Nine mice were anesthetized with tribromethanol (500 mg/kg) and perfused through the heart with ice-cold heparinized (15,000 IU/l), phosphate-buffered saline (PBS) (pH 7.4) for 2 min, followed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 15 min. The brains were removed and postfixed in the same fixative overnight and rinsed in cacodylate buffer. The pineal glands were postfixed at room temperature in 2% OsO_4 in 0.2 M sodium-cacodylate buffer (pH 7.4) for 3 h and stained *en bloc* with 2% uranyl acetate in maleate buffer for 1 h. The tissue was then dehydrated in a graded ethanol series and embedded in Epon. Survey sections of 1 to 2 μm of thickness were cut. Thin sections with a gray interference color were poststained with uranyl acetate and lead citrate and viewed by a Philips 300 electron microscope operated at 80 kV.

Cytochemical Demonstration of Acid Phosphatase

Twenty mice were killed by ether inhalation and the pineal glands immediately removed and fixed by immersion in ice-cold 2% purified

glutaraldehyde in 0.1 M sodium-cacodylate buffer for 30 min to 19 h. Following fixation, the pineals were rinsed in the same buffer, embedded in 7% agar, and chopped into 50 μm thick sections. The sections were incubated for 1.5 h at 37°C for acid phosphatase activity according to the method of Barka and Anderson (1962) with 0.01 M β-glycerophosphate and 0.002 M $PbNO_3$ in 0.02 M Tris-maleate buffer (pH 4.8). In 4 animals, 0.01 M NaF was added to the incubation medium as an inhibitor of acid phosphatase activity. After incubation, the pineal glands were fixed in osmium and processed for electron microscopy as described above.

Horseradish Peroxidase Uptake

Forty mice received, via the tail vein, 20 mg horseradish peroxidase (HRP) (Sigma type II) dissolved in PBS. The tracer was allowed to circulate for 40 sec, 1, 5, 30, 60, or 120 min before the animals were anesthetized and fixed by vascular perfusion as described above. The pineals were chopped into 50 μm thick sections and incubated for peroxidatic activity as previously described (Møller et al., 1978b). This was followed by osmification and processing for electron microscopy as described above.

Immunohistochemistry

Three animals were anesthetized with tribromethanol and perfused through the heart with ice-cold heparinized PBS for 2 min, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, postfixed in the same fixative for 24 h, cryoprotected for 2 d in 25% sucrose, frozen in crushed dry ice, sectioned in a cryostat at a thickness of 18 μm, and thaw-mounted on Superfrost Plus® glass slides. The sections were washed 3 × 5 min in PBS and incubated for 30 min in 5% normal swine serum. They were then incubated for 12 h in mouse anti-rat CD68 (Serotec, Oxford, #MCA341R) diluted to 5 μg/mL in PBS with 0.25% bovine serum albumin and 0.1% TritonX-100 (PBS-BT) and anti-mouse/rat IA (Serotec, #MCA46R) diluted to 10 μm/mL in PBS-BT. The anti-mouse/rat IA antibody recognizes the IA region of MHC-proteins. The sections were washed 3 × 10 min in PBS-BT and incubated for 60 min at room temperature in rabbit anti-mouse IgG coupled to Alexa 568 (Molecular Probes, Eugene, Oregon, USA, #A11061) diluted 1/100. The sections were cover slipped with Aquamount® (DakoCytomation, Copenhagen, Denmark). The sections were viewed by a Zeiss Axioplan microscope equipped with epifluorescence and interference filters. Images were taken with an AxioCam MRm digital camera and transferred to a computer with AxioVision 4.1 software. Adjustment of contrast was done in Adobe Photoshop 7.0.

RESULTS

Light Microscopy of 2 μm Thick Sections

Large, lightly stained pinealocytes with euchromatin were clearly seen in the Epon-embedded semi-thin sections stained with Toluidine blue (Figure 1). Interstitial cells were identified between the pinealocytes. They exhibited a triangular shape, and both the cytoplasm and nucleus were darker than those of the pinealocytes (Figure 1). Darkly stained triangular cells were seen in the perivascular spaces. They exhibited an interstitial cell-like morphology with dense nucleus and cytoplasm (Figure 1).

Transmission Electron Microscopy (TEM)

Pinealocytes were easily identified in TEM by their electron lucent cytoplasm containing many mitochondria and ribosomes and a nucleus with euchromatin (Figure 2A). The interstitial cell had a more electron-dense cytoplasm (Figure 2D) and processes that extended between the pinealocytes.

Perivascular phagocytes in the perivascular spaces exhibited interstitial cell-like morphology (Figure 2C). Their cytoplasm was electron-dense and contained many lysosomes (Figures 2C and 2D). After exposure to HRP for 1 to 5 min, the perivascular phagocytes showed a high uptake of the tracer that entered the lysosomal system (Figure 2C). In contrast, the pinealocytes exhibited a slower HRP uptake via coated vesicles of the

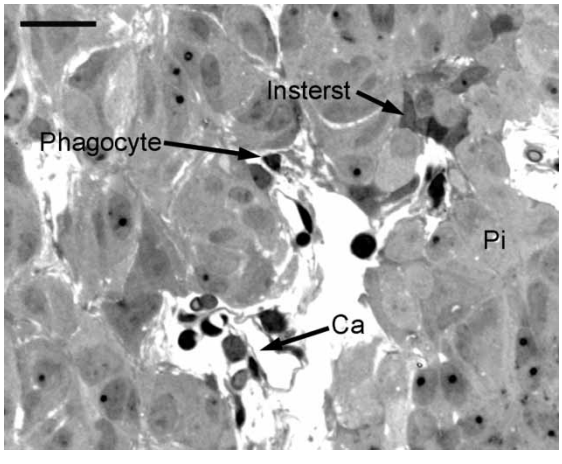

FIGURE 1 Photomicrograph of a 2 μm thick Epon-embedded section of the mouse pineal gland stained with Toluidine blue. A triangular dark interstitial cell (*Insterst*) is seen between the pinealocytes. A phagocyte (*Phagocyte*) is located in the perivascular space. Ca = capillary, Pi = pinealocyte. Bar = 20 μm.

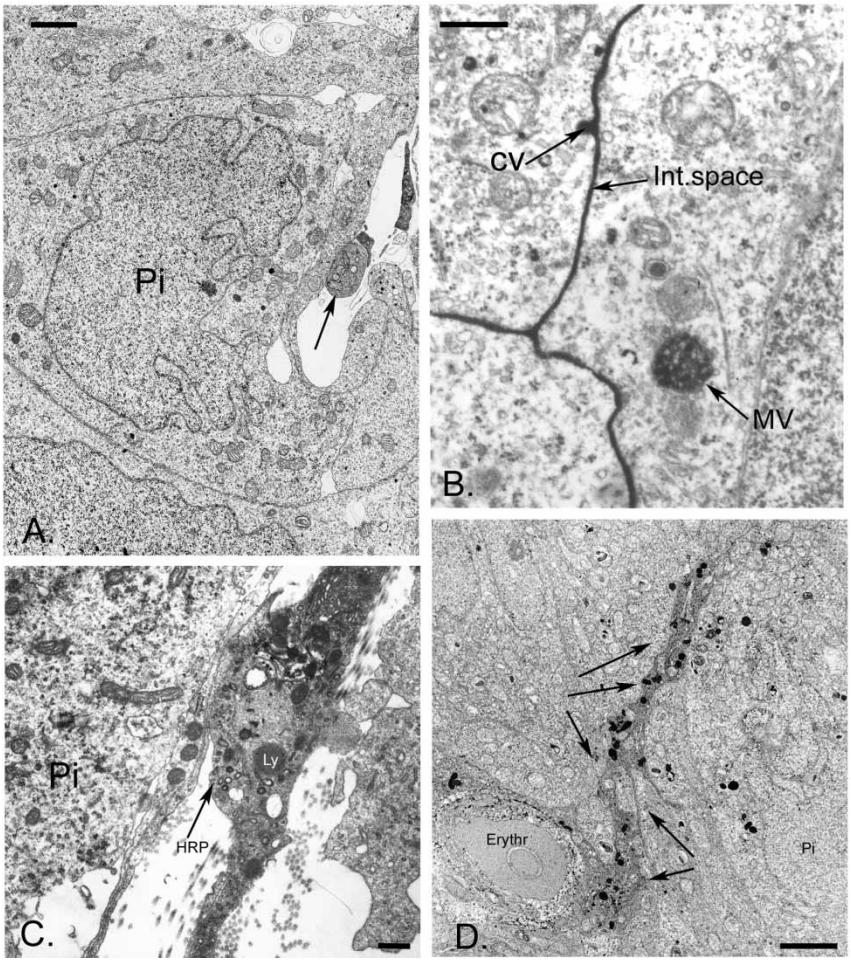

FIGURE 2 Electron micrographs of the mouse pineal gland. ***A:*** interstitial cell process (arrow) with electron dense cytoplasm in the intercellular space between pinealocytes (*Pi*) that display an electron lucent cytoplasm and a nucleus with euchromatin. ***B:*** horseradish peroxidase (*HRP*)-filled intercellular space (*Int. space*) between two pinealocytes. A coated vesicle (*CV*) is seen filled with HRP. An HPR-filled multivesicular body (*MV*) is seen in the pinealocyte in the right site of the picture. ***C:*** perivascular phagocyte with many HRP-filled vesicles (*HRP*) and many lysosomes (*Ly*) located in the perivascular space of the mouse pineal. ***D:*** electron micrograph of a perivascular phagocyte (*arrows*) with a process penetrating from the perivascular area into the pineal parenchyma between the pinealocytes. The sections were reacted for acid phosphatase, and the lysosomes of the perivascular phagocytes exhibited acid phosphatase reactivity. Erythr = erythrocyte in a capillary. Bars = 1 μm (*A, C*), 0.5 μm (*B*), and 3 μm (*D*).

cell membranes (Figure 2B). After detachment from the cell membrane, the tracer-filled vesicles lost their coat and became smooth vesicles, which merged with the primary lysosomes. The tracer was also found in multivesicular bodies (Figure 2B). After 30 min and 60 min, the tracer was only found in lysosomes. After 120 min, the tracer was only occasionally observed in the perivascular phagocytes and pinealocytes.

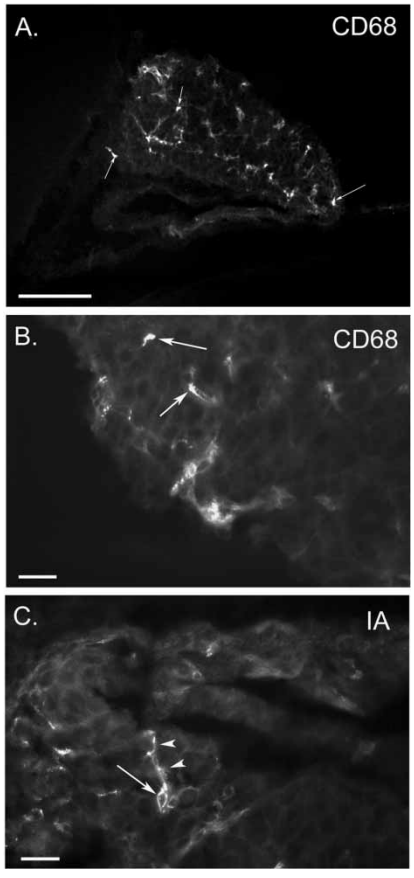

FIGURE 3 Fluorescence micrographs of a mouse superficial pineal gland in which macrophages have been immunohistochemically visualized by use of a monoclonal antibody against CD68 and the I-A region of the MHC-molecule. ***A:*** Anti-CD68 immunohistochemical reaction. Several immunoreactive cell bodies (*arrows*) are seen in the perivascular and interstitial spaces of the gland. Many thin immunoreactive processes are also present. ***B:*** Higher magnification of CD68 immunoreactive cells (*arrows*) in the superficial mouse pineal gland. ***C:*** Immunohistochemical reaction for the I-A region of the MHC-molecule in the mouse superficial pineal gland. An immunoreactive cell (*arrow*) with a long process (*arrowheads*) is present in a perivascular space. Bars = 0.2 mm (*A*), 15 μm (*B* and *C*).

The perivascular phagocytes (Figure 2C) exhibited a strong acid phosphatase reaction in the lysosomal system. The reaction was weaker in the pinealocytes, and the number of lysosomes was lower.

Fluorescence Immunohistochemistry

Triangular immunopositive CD68 (Figures 3A and 3B) and I-A (Figure 3C) perivascular cells were identified; the cells were endowed with long processes localized in the perivascular spaces (Figure 3C). The staining for the I-A region of the MHC protein was mostly confined to

the cell membrane (Figure 3C). These cells were present in the superficial pineal, the pineal stalk, and deep pineal gland.

DISCUSSION

This study demonstrates the presence of the perivascular phagocyte in the mouse pineal gland and suggests that this cell might be generally present in the mammalian pineal gland. The characteristic high uptake of an exogenous HRP tracer as observed in this study on the mouse is in accord with a previous HRP tracer study done in the rat by Kaur and coworkers (1997a), who, after injection of the tracer, observed its high phagocytotic uptake in these cells. This cell type is in a good anatomical position for phagocytosing material brought to the pineal via the blood stream, because it is located in the perivascular space, close to the fenestrated capillaries lacking a blood-brain barrier (Møller et al., 1978b).

Several immunohistochemical studies on the rat performed with mouse monoclonal antibodies raised against leukocyte-membrane markers have characterized the perivascular phagocytes as macrophages/microglial cells and antigen-presenting cells (Pedersen et al., 1993, 1997; Sato et al., 1996; Jiang-Shieh et al., 2003; Mukda et al., 2005). Classical immunostimulation of rats with cell-wall components from gram-positive (LTA) and gram-negative bacteria (LPS) has resulted in a proliferation of perivascular phagocytes (Jiang-Shieh et al., 2005). Proliferation is also observed after exposure of rats to a non-penetrative blast (Kaur et al., 1997b) or a high-altitude atmosphere (Kaur et al., 2002).

The perivascular phagocytes of the rat pineal gland are able to synthesize cytokines, including interleukin-1β (Tsai and McNulty, 1999), which can influence pineal indole metabolism (Tsai et al., 2001). As antigen-presenting cells, the perivascular phagocytes might present MHC class II proteins to CD4+ and CD8+ lymphocytes. Lymphocytes are present in, or in close apposition to, the pineal glands of several species, *e.g.*, the chicken (Cogburn and Click, 1983), rat (Uede et al., 1981), and mouse (Abe et al., 1971). It is also of interest that lymphocytes are present in pineal germinomas (Nitta et al., 1995).

The retinal S-antigen (arrestin) is present in the pineal glands of mice (Korf et al., 1990) and other mammals (Korf et al., 1985), and presentation of S-antigen to T-cells initiates a cascade resulting in severe experimental pinealitis and autoimmune uveoretinitis (de Kozak et al., 1981; Mochizuki et al., 1985). If S-antigen is phagocytosed by the perivascular phagocytes but not presented to T-cells, the perivascular phagocytes might function as a sentinel system in protecting the pineal, choroids, and retina from inflammation.

ACKNOWLEDGMENTS

Supported by the Danish Medical Research Council, grant number 22-02-0288, the Carlsberg Foundation and the Lundbeck Foundation. The authors are grateful to Mrs. Ursula Rentzmann for expert technical assistance.

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